EFFEECTS OF MEDIA COMPONENT ON ARACHIDONIC ACID PRODUCTION BY CANDIDA KRUSEI UNDER SUBMERGED CONDITION

ABSTRACT

Arachidonic acid (ARA) is a long chain polyunsaturated fatty acid (PuFA) of the omega-6 class (5,8,11,14-eico-satetraenoic acid). It plays an important role in the structure and function of biological membranes (Yu et al., 2002), which also as an essential fatty acid in human nutrition and a biogenetic precursor. The aim of this study is to develop an optimum media component (glucose, potassium nitrate (KNO_3), potassium phosphate monobasic (KH_2PO_4), and glutamate) to yield the highest production of ARA by *Candida krusei* under submerged condition. The media components are studied at different levels which are from 10 to 80 g/L for glucose, 1 to 5 g/L for KNO_3, 1 to 5 g/L for KH_2PO_4 and 0 to 0.8 g/L for glutamatewhich are determined using Design Expert. The total ARA composition will be determined by gas chromatography (GC) with capillary column 0.25 mm x 30 m x 0.25 µm, using helium as the carrier gas and at initial column temperature at 185 °C which then will increase at a rate of 3.5 °C min^{-1} to 235 °C.From this study, the expected optimum conditions that will be achieved is with glucose concentration, 10.0 g/L, KH_2PO_4 concentration, 1.0 g/L, KNO_3 concentration 1.0 g/L and concentration of glutamic acid, 0.8 g/L which resulted in maximum production of ARA(1.39 g/L). In a conclusion, the optimization of media to produce high amount of ARA using microorganism is a suitable alternative way to produce ARA which benefits everyone besides getting ARA from porcine liver which is prohibited in certain community such as the Muslims and vegetarians.

ABSTRAK

Asid arakidonik (ARA) adalah rantaian asid lemak tak tepu (PUFA) yang dikelaskan sebagai omega-6 (5,8,11,14-eico-satetraenoic asid). Ia memainkan peranan penting dalam struktur dan fungsi membran biologi (Yuet al., 2002), yang juga sebagai asid lemak yang penting dalam pemakanan manusia dan pelopor biogenetik. Tujuan kajian ini adalah untuk membangunkan satu komponen media optimum (glukosa, kalium nitrat(KNO_3), kalium fosfat yg berdasar satu(KH_2PO_4), dan glutamat) untuk menghasilkan pengeluaran tertinggi ARA oleh *Candida krusei*. Komponen media dikaji pada tahap yang berbeza iaitu 10 hingga 80 g/L untuk glukosa, 1 hingga 5 g/L bagi KNO_3, 1 hingga 5 g/L untuk KH_2PO_4 dan 0 hingga 0.8g/L untuk glutamat. Komposisi ARA di dalam sampel dikaji menggunakan gas kromatografi(GC) dengan turus kapilari 0.25mm x 30m x 0.25μm, menggunakan helium sebagai gas pembawa dan pada suhu awal 185° C yang kemudiannya akan meningkat pada kadar sebanyak 3.5 °C min^{-1} hingga 235 °Cmin^{-1}. Hasil daripada kajian ini, keadaan optimum yang akan dicapai adalah dengan kepekatan glukosa, 10.0 g/L, kepekatan KH_2PO_4, 1.0 g/L, kepekatan KNO_3, 1.0 g/L dan kepekatan asid glutamik, 0.8 g/L dimana pengeluaran maksimum ARA dicapai(1.39g/L). Sebagai kesimpulan, pengoptimuman media untuk menghasilkan jumlah ARA yang tinggi menggunakan mikroorganisma adalah satu idea alternatif yang sesuai untuk menghasilkan ARA dan juga dapat memberi manfaat kepada semua lapisan masyarakat terutama kepada penganut Islam yang diharam untuk memakan khinzir.

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INTRODUCTION

1.0 Background of study

The role of yeast in biotechnology had been discovered for a long time. Other than used in production of food and beverages, yeasts also play important role in pharmaceutical field. They are involved in production of medicines, dietary supplements and probiotics. As the importance of yeast in biotechnology is expanding, huge amounts of yeast are cultivated nowadays. Despite the fact that yeast can cause many diseases, researchers continue to investigate other angelic side of yeast that can be developed for humans' sake. One of a newly discovered application of yeast is found in production of fatty acid, such as linoleic acid, alpha-linolenic acid, and arachidonic acid **(**Yazawa et al., 2009).

Arachidonic acid (ARA), (5-cis,8-cis,11-cis,14-cis-eicosatetraenoic acid or 20:4(n-6)) is a long chain polyunsaturated fatty acid (PuFA) of the omega-6 class which plays important roles in the structure and function of biological membranes (Yamada et al., 1989). The classification of fatty acids, where ARA was classified under polysaturated of omega-6 class is shown in Figure 1.1. It is one of polyunsaturated fatty acids, an essential fatty acid in human nutrition and a biogenetic precursor of several key eicosanoid hormones, such as prostaglandins, thromboxanes leukotrienes and pharmacologically active metabolites (Aki et al., 2001). On account of its unique biological activities, ARA plays an important role in the growth and brain development of infant. Various physiological functions of ARA which are for protection of gastric mucosa, treatment of skin psoriasis, reduction of fatty liver, killing of tumor cells, and improvement of lipid metabolism of cirrhotic patients which cirrhotic is defined as a chronic interstitial inflammation of any tissue or organ.

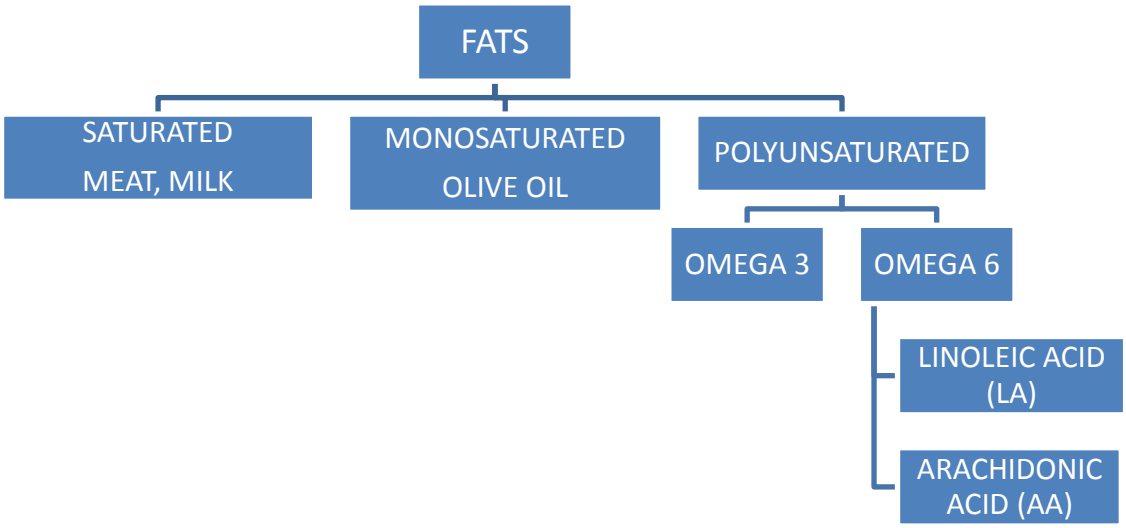

Figure 1.0 Classification of fatty acids

Omega-6 class fatty acids are very crucial in many ways. Firstly, it is crucial in growth of human. Omega 6 plays an important role in cell growth, and is thus essential for brain and muscle development. The omega 6 arachidonic acid (ARA) is for this very reason added to most infant formulas. Both brain development and muscle development are critical for infants. The growth benefits of omega 6 also explain the great interest that body builders and top athletes have in omega 6 consumption.

Secondly, it acts as a hormone like messengers. Messengers made from play an important role in swelling, pain, blood thinning, blood vessel spasms and accumulation of inflamed cells. You may wonder why pain or inflammation is considered a benefit. The answer is that pain is an important signal that prevents further injury, and inflammation is a trigger for our immune system.

Arachidonic acid is found in large quantities in diary and animal meats and fats. As mentioned, it's highly inflammatory, and if not balanced with omega 3 will cause chronic inflammation diseases such as heart disease, arthritis, obesity, and diabetes, as well as many mental disorders.

1.1 Problem Statement

The main sources of arachidonic acid are from plants and animals which are from fish and swine. However, the amount of arachidonic acid (ARA) produce from the animal and plant sources yield are very low and because of the low yield, it is difficult to industrialize. Furthermore, the process is more complicated as the removal of cholesterol, odours and tastes is quite difficult. By considering the geographical location and fishing season, marine resources could be very limited and unstable. In this matter, the source of ARA is very difficult and limited, therefore alternative sources are being sought.

Apart from that, swine became one of the major producer of ARA which became an issue because porcine is a *non-Halal* animal and is prohibited in certain religion and vegetarians. With the rising consumer demand for a '*Halal*' integrated lifestyle, it is needed to find alternatives in producing products that can fit '*Halal*' standards. Besides that, ARA that is produced from marine livings gives bad and uneasy odor. In this matter, most people definitely will not purchase or even want that ARA.

1.2 Research Objective

The main objective of this study is to study the effect of media components (glucose, potassium nitrate (KNO_3), potassium phosphate monobasic (KH_2PO_4), and

glutamate) on production of ARA by *Candida krusei* under submerged condition. Besides, the aim of the study is to be able to produce quality production of ARA that is suitable to all communities. The measureable objectives are:

a) To study the effect of glutamic acid in the production of ARA
b) To study the effect of carbon source
c) To study the effect of nitrogen source
d) To study the effect of phosphorus source.

1.3 Scope of study

In order to achieve the objectives, the effect of the media components was done in this study. In order to design the variation of the media components, response surface methodology (RSM) was used because RSM is the best and suitable method for optimizing unknown variables. The selected media component variables were glucose, potassium nitrate (KNO_3), potassium phosphate monobasic (KH_2PO_4) and glutamate. Each of the media components were varied to some concentration ranges which are as follows:

1) glucose concentration from 10g/L to 80 g/L ,

2) potassium nitrate (KNO_3) from 1g/L to 5 g/L,

3) potassium phosphate monobasic (KH_2PO_4) from 1g/L to 5 g/L

4) glutamate from 0 g/L to 0.8 g/L

Gas chromatography was used to analyze the concentration of each the selected media components to find the most suitable condition of the media components to produce highest amount of arachidonic acid.

1.4 Significance of study

Why is this study significant? We can look into this through three views; the implications of this study towards ourselves, communities, and a nation as a whole. As an individual, it is almost as impossible for anyone to neglect matters concerning and leaning towards a good health. Through this study, that objective is within reach as benefit of arachidonic acid linking to human physiological and biological functions is to be explained briefly here. The physiological functions of ARA that has been reported varies with every cases reported along with it. Recently, a new function of ARA was reported; *arachidonylethanolamide,* also called anandamine, which may function as a natural ligand for the cannabinoid receptor, which is expressed in areas of the central nervous system that contribute to the control of memory, cognition, movement and pain perception (Higashiyama et al, 2002). ARA is also being studied as to how its deficiency could greatly reduce the chances of improvements for the first year growth of an infant.

This information is very valuable to human race as a whole as we look to go beyond in health sciences.

Besides, we do believe one healthy person could influence one community's lifestyle, leaning them towards better and healthier life. Yes, there is a bad side to everything. In terms of its' *haram* or *halal* point of religious view, this may not be able to be consumed by some consumer; which in this particular case, the Muslims. This is another turning point of why this particular study is conducted. The experiment is designed to eliminate uncertain variables which includes its' status with the Muslims. One of the objectives of this study is to have ARA usable by all, functioning to its' potential regardless of any ones' religious view. As to how far the study has gone, it is found that sources of marine life; which contributes to ARA, emits certain odour to which at a certain point can be regarded as extreme discomfort. Its' alternative, porcine liver, however is; as stated, not usable by all in particular the Muslims.

Everything starts with a healthy start. Looking from the governments' view, a healthy country has higher chances of having better productivity both domestically and internationally. This indirectly also boost another sector of the nation; the economics. As to the implications stated so far, this study could implicate such effects both directly and indirectly towards bigger parties investing in this study of ARA. Other than that of what had been stated, another important sector are also boosted which is education. As we look more into the study of these microorganisms, more benefits and dangers are yet to be unfolded. This gives the nation a chance to go beyond in this field of study. To

conclude this section, the significances of this study go beyond any particular individuals, communities or a nation as to how it may affects them directly or indirectly. There are always a good side to having studies leaning towards the good of human health.

CHAPTER 2

LITERATURE REVIEW

2.0 Arachidonic Acid (ARA)

Arachidonic acid (ARA), $C_{20}H_{32}O_2$, is a carboxylic acid which categorize in a polyunsaturated acid which contains 20 carbon chains. Figure 2.2 illustrates the structure of ARA.

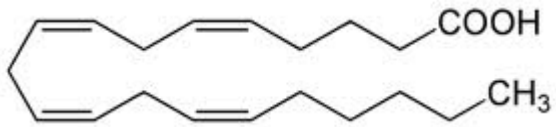

Figure 2.0 Arachidonic acid structure

ARA is one of a common unsaturated fatty acid. The unsaturated refers to the presence of one or more double bonds between carbons in the structure. The double bonds results in introduction of a kink in the molecule shape, which makes it difficult to

pack together. The intermolecular interactions are weaker, and hence the melting points are much lower than saturated fatty acid. Table 2.0 shows basic properties of ARA.

Table 2.0 Arachidonic acid properties

ITEM	INDICATOR
Molecular Weight	304.47 g/mol
Molecular Formula	$C_{20}H_{32}O_2$
Melting point	-49 °C
Boiling point	170 °C
Specific gravity	0.922 g/mL
Storage temperature	- 20°C
Solubility	ethanol: ≥10 mg/ml
Form	oil
Color	colorless to light yellow
Water Solubility	practically insoluble

(Source: http://www.chemicalbook.com)

2.1 Sources of Arachidonic Acid

At the present time, the oil extracted from porcine liver is generally used as the main source of ARA. The main sources of ARA are getting increase where they can be found in bovine and porcine liver, adrenal glands and sardines (Yu et al., 2002).

However, the ARA content per unit dry weight of these sources is only 0.2% (Bajpai et al., 1991) which is very low and very difficult to be industrialized (Yuan et al., 2002). Furthermore, swine is forbidden for certain religion and vegetarians. Furthermore, the process is more complicated as the removal of cholesterol, odours and tastes is quite difficult to remove from fish oil concentrates and animal sources (Jareonkitmongkol et al., 1993). According to Yongmanitchai and Ward (1989), by considering the geographical location and fishing season, marine resources could be very limited and unstable. ARA also can be produced from microorganisms.

The interest in arachidonic acid (ARA) and other polyunsaturated fatty acids (PUFAs) inspired the search for new sources of these PUFAs. Several microalgae were shown to contain high proportions of long-chain PUFAs (LC-PUFA) among their fatty acid (Bigogno et al., 2002). According to Araki et al. (1990) in *Gracilaria sp.*, the proportion of ARA can be as high as 60 % of total fatty acids, however, the dry weight content does not exceed 0.2 %. Araki (1990) and Cohen (1990) produced ARA from *Porphyridium cruentum* and found out that the yield of AA was high only when the growth is slowed (Cohen, 1990). Bigogno et al. (2002) found that the fresh-water green microalgae *Parietochloris incisa* is the only ARA-rich vegetal organism. In addition, Higashiyama et al. (1998) reported that the isolation of *Mortierella alpina* 1S-4 is a potent producer of ARA and the fundamental culture condition. The sources of ARA were summarized in the Table 2.1.

Table 2.1 Sources for ARA production

Source	Reference
Algae *Parietochloris incisa*	Bigogno et al. (2002) Cheng- Wu et al. (2002)
Algae *Porphyridium cruentum*	Cohen (1990); Araki et al. (1990)
Moss *Leptobryum pyriforme*	Hartmann et al. (1986)
Fungi *Mortierella alpina*	Bajpai et al. (1991); Streekstra (1997); Park et al. (1999); Eroshin et al. (2000); Yuan et al. (2002); Yu et al. (2003); Zhu et al. (2005)
Fungi *Mortierella alpina* 1S-4	Higashiyama et al. (1998)
Fungi *Diasporangium sp.*	Cheng et al. (1999)
Fungi *Mortierella elongata*	Bajpai et al. (1991); Cheng et al. (1999);

In addition, the productivity of ARA production by several types of microorganisms, algae, plants and fungi can be summarized in Table 2.3 below.

Table 2.2 ARA productivity by microorganisms (Saelao et al.,2010)

Microorganism	ARA productivity
Bacteria	
Antarctic bacteria strain 651	0.01
Aureispira maritima TISTR 1715	14.69
Fungi	

Achlya sp. ma-2801	14.4
Mortierella alpina	267.14
M. alpine	967.50
M. alpina ATCC 32222	535.84
M. alpina I49-N18	758.33
M. alpina M18	201.43
M. alpina ME-1	1541.54
M. alpina 1S-4	1362.50
M. alpina strain ZQ 9998	200
Algae	
Nannochloropsis oceanica	1.83
Oocystis sp.	0.12
Parietochloris incisa	70.18
Pavlova sp.	0.11
Phaeodactylum tricornutum	0.99
Rhodomonas baltica	0.02
Tetraselmis sp.	0.15
Lower plant	
Marchantia polymorpha	4.38
Physcomitrella patens	3.06

2.2 Microbes

Candida krusei is a budding yeast which is classified in fungi species. It is classified in Saccharomycetaceae family where it is inherently resistant to fluconazole and as a frequent cause of fungemia in patients with hematologic malignant neoplasms. It also acts as a human pathogen during the last two to three decades. It is generally elongated and have the appearance of "long grain rice". *Candida krusei* grows at a maximum temperature of 43 to 45°C. It also has been isolated from a large variety of natural habitats such as the atmosphere, fruits, sewage, silage, soil, foods which including dairy and meat products, pickles, sugar and syrup-based products, wines and beer. Hence, it is widely distributed in nature and considered to be a facultative saprophyte. It is also found in chickens and seagulls (Samaranayake et al., 1994). It is widely used in chocolate production. According to Liu et al. (2004), it also involved in production of glycerol as it has the ability to ferment glucose into glycerol. *Candida krusei* is resistant to fluconazole and is the cause of fungemia in human (Abbas et al., 2000). Figure 2.3 illustrates the *Candida krusei*.

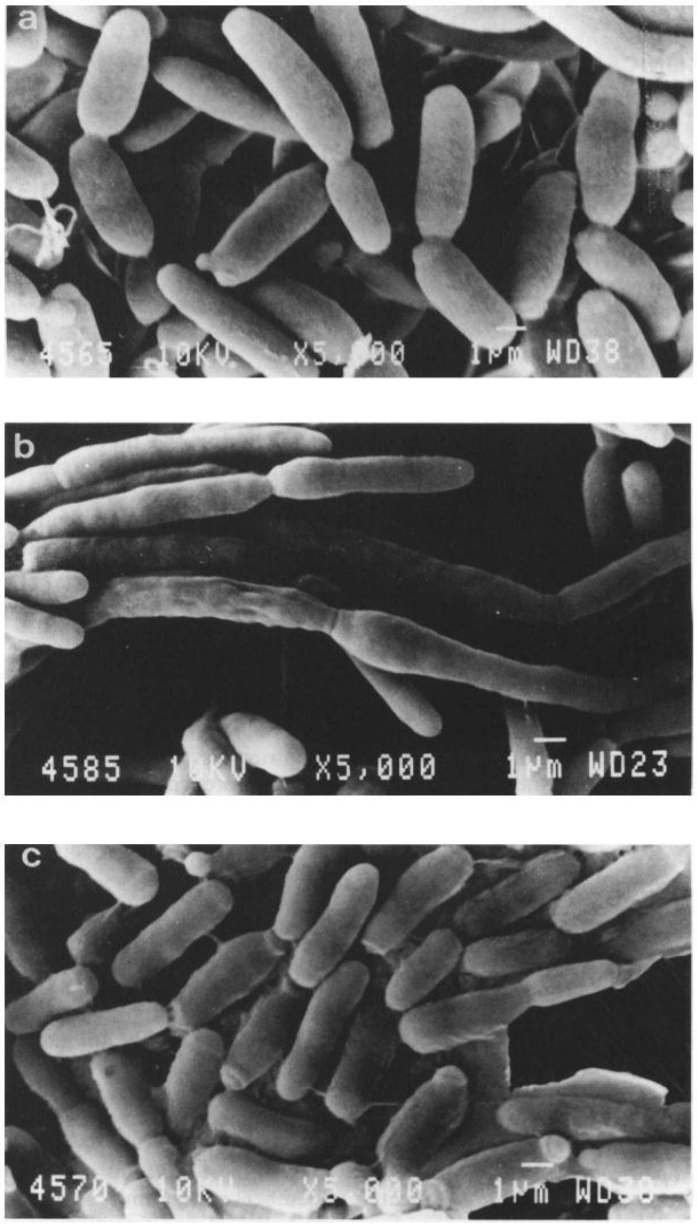

Figure 2.1 Scanning electronmicrographs of three oral isolates of *Candida krusei*

2.3 Optimum Condition for Cell Growth

Culture media generally contain a source of carbon, nitrogen and vitamins. Glucose is the most widely utilizable carbon source, and hence is the most commonly used in growth media. Fructose and mannose are the next most commonly utilized sugars by bacteria and are found in media from natural sources. Sucrose also may be used in some media. Some species can use other more complex carbohydrates such as starch, glycerol and maltose, due to special enzymes they release. This glucose can be respired (used to provide energy) in a similar way to respiration in most other organisms. Research from Bajpai et al, (1991) showed that growth of fungi *Mortierella alpina* was very good by using glycerol and linseed oil. However, the growth was found to be very poor with lactose, starch and sucrose as carbon source and moderate with glucose, fructose and maltose.

A culture medium provides all of the nutrients and the energy source required by the organism. Culture media can come in liquid, semisolid or solid forms. The liquid medium that lack of solidifying agent is called a broth medium whereas the solid or semisolid medium is prepared by adding a solidifying agent like agar into the broths. According to the US Pharmacopeia, agar can be defined as a hydrophilic colloid extracted from certain seaweeds of the Rhodophyceae class. It is insoluble in cold water but soluble in boiling water. Because of these properties, microorganisms can be cultivated at the temperature of 37.5^{o}C without fear of the medium liquefying. Semisolid medium contains a concentration of less than 1% agar (Cappuccino and Sherman, 1998). Semi-solid media can also be used in fermentation studies, in determining bacterial

motility, and in promoting anaerobic growth. A completely solid medium requires an agar concentration of about 1.5 % to 1.8 %. Solid media are usually used for the surface growth of microorganisms in order to observe colony appearance, for pure culture isolations, for store of cultures, and to observe specific biochemical reactions. Solid medium is usually used as agar plate, agar slants and agar deep tubes.

Agar plate is the agar that poured into a petri plate. Compared to broth that contains high concentration of microorganism, petri plates allow observation, easy access (microorganism can be counted easily), and isolation, but are prone to environmental contamination and desiccation. Petri dish has to be inverted during the incubation to prevent any condensed moisture from falling on the surface of the agar so as to acquire a clear observation on microbes grown (Lester and Birkett, 1999). Bacteria may be grown on a semi-solid agar medium and also in liquid media, in which it grows best.

Bacteria is an organism which can be found everywhere, whether in water, air or land, depending on their species. *Candida krusei*, an ascomycetes, is an environmental yeast that is usually present in milk products, beer and bird feces. This yeast is known for its use in chocolate production. *C. krusei* also is an osmophilic yeast that can ferment glucose into glycerol (Liu et al., 2004). To culture this microorganism, 10% of pre-cultured seed was transferred into fermentation medium and then followed by 5-day incubation at 35 °C and 150 rpm (Chen et al., 2006).

In this study, the cultures for ARA production, glucose is most frequently used as the carbon source. Aki et al. (2001) investigated various carbon sources for ARA production using strains of *M. alpina* and *M. alliacea*, respectively, and found that glucose was suitable for ARA production which high glucose concentration induced the formation of filamentous morphology (Higashiyama et al. 2002). In order to obtain a higher product yield, optimization of the trace element composition in the medium is also important besides the fundamental medium composition. Phosphorus, potassium, sulfur, calcium, sodium, iron, and magnesium are the major inorganic constituents of fungi where these minerals should be supplied sufficiently, and optimization of the additional concentration is important. Therefore, various groups have attempted to enhance ARA productivity by optimization of the amounts of minerals added.

2.4 Application of Arachidonic Acid

In recent years, arachidonic acid (ARA) has been widely used in phytoalexins in plants which for preventing plant diseases. Nowadays, the main sources of ARA are in number of fields such as pharmaceuticals, cosmetics and agriculture (Eroshin et al., 1995). ARA is a long chain polyunsaturated fatty acid (PuFA) of the omega-6 class (5,8,11,14-eico- satetraenoic acid) which known to have very important roles in the structure and function of biological membranes (Yu et al., 2002). It is also an essential fatty acid in human nutrition and a biogenetic precursor of several key eicosanoid hormones, such as prostaglandins, thromboxanes leukotrienes and pharmacologically active metabolites (Aki et al., 2001). Higashiyama et al. (2002) also stressed on the

various physiological functions of ARA, for example protection of gastric mucosa, treatment of skin psoriasis, reduction of fatty liver, killing of tumor cells, and improvement of lipid metabolism of cirrhotic patients.

Besides that, ARA plays an important role in the growth and brain development of infant (Koletzko et al., 1996) where it works as a component of human milk, which is necessary for the neurological and neurophysiological development. Eicosanoids derived from long chain PUFAs including arachidonic acid play a key role in modulating inflammation, cytokine release, immune response, platelet aggregation, vascular reactivity, thrombosis, and allergic phenomenon. Formula supplemented with a combination of docosahexaenoic acid (DHA) and ARA proved to be of benefit in mental develop ment, visual function, cognitive development and blood pressure, well beyond the period of supplementation into early childhood. The LC-PUFAs including arachidonic acid are not only important in infant formulas and dietary supplements but also in functional foods, beverages and cosmetics, which supports the idea that these products provide a benefit beyond basic nutrition (Koletzko et al., 1989).

2.5 Recent Study

Fermentation of microorganisms is one of the best processes for the production of ARA. Process optimization in general could be achieved either by empirical or statistical methods. Empirical methods adopt a one factor at a time approach, in which one factor is varied at a time while all others are kept constant, which may lead to

misinterpretation of results, especially when interaction between different factors is prevalent. Furthermore, such methods suffer several set backs, such as being more time consuming, requiring large number of experiments, being uneconomical, and most importantly, lacking the mutual interactions among the variables. Statistical methods provide an alternative methodology to optimize a particular process by considering the mutual interactions among the variables and give an estimate of the combined effects of these variables on the final results.

Response surface methodology (RSM), a powerful tool and an efficient mathematical approach, is useful for developing, improving and optimizing the fermentation process and is used extensively where several independent variables potentially influence the response of a process. RSM has been widely used in the optimization of the fermentation process such as media components for the production of enzyme, exopolysaccharide, other metabolites, biomass, and for predicting the growth rate of Bacillus cereus. RSM is an effective tool for optimizing the process when many factors and interactions affect the desired response. The interactive effect of different media components such as glucose, KH_2PO_4, and KNO_3 on the production of biomass, total lipid and arachidonic acid was investigated in M. alpina and the reaction conditions for maximized ARA content were optimized using RSM. (Nisha et al., 2008).

ARA exists widely in the animal kingdom, and has been isolated from lipids extracted from the adrenal gland and the liver of animals. However, because such organs contain ARA in small amounts, isolation from these organs is insufficient to meet the demand for ARA. Methods to produce ARA by cultivation of various microorganisms

capable of producing ARA have been proposed. In terms of cultivation methods, there are two types: the submerged culture and the solid-state culture. In order to industrialize PUFA production, the submerged culture seems to be the better choice. For one, the scaling up of cultivation and biomass recovery is easier, and ARA yield reported in the solid-state culture is less than that in the submerged culture. Moreover, in the case of solid-state culture, the cultivation period is more than 20 days because of the low growth rate. In terms of industrial production, the improvement of ARA yield and the reduction of cultivation time have an impact on the production cost.

In general, sufficient mass transfer and oxygen supply are required to obtain high productivity in aerobic fermentation. In the case of fungal fermentation, the medium composition not only influences the productivity, but also may induce a change in mycelia morphology as a side effect. The morphology has a strong effect on the physical properties of the fermentation broth, and causes numerous problems in large fermenter with respect to gas dispersion, mass and heat transfer, and homogenization. Thus, mycelia morphology is often considered to be one of the key parameters in industrial fermentation.

For the commercial production of ARA, a high biomass concentration is required for high productivity because ARA is an intracellular product. Besides, ARA production requires adequate oxygen, because PUFAs are formed by enzymatic desaturation that comprises oxygenation. Thus, adequate oxygen supply by means of agitation, aeration, and morphological control is the key factor for obtaining a higher ARA content in the cells.

CHAPTER 3

RESEARCH METHODOLOGY

3.0 Chemicals

The chemicals, the brand of chemicals and the usage of the chemicals used in this study are shown in Table 1 as follows:

Table 3.0 List of chemicals and usage

CHEMICALS	ORIGIN/BRAND	USAGE
Glucose anhydrous	Aldrich	Inoculums
Yeast extract agar	Fluka	Microorganism cultivation
Yeast extract powder	Sigma	Inoculums

Potassium nitrate	Sigma	Fermentation
Potassium phosphate monobasic	Sigma-Aldrich	Fermentation
Zinc sulphate heptahydrate	Sigma-Aldrich	Fermentation
Calcium chloride dihydrate	Sigma	Fermentation
Magnesium sulphate heptahydrate	Sigma-Aldrich	Fermentation
Iron(lll) chloride hexahydrate	Sigma	Fermentation
Cupric sulphate	Sigma-Aldrich	Fermentation
Hydrochloric acid	Sigma	Biomass production
Hexane	Sigma-Aldrich	Lipid extraction
Chloroform	Sigma-Aldrich	Lipid extraction
Methanol	Sigma-Aldrich	Growth media
L-glutamic acid	Sigma	Growth media
Standard Arachidonic Acid	Sigma	

3.1 Equipments

The equipments and the usage of the equipments that were used in this study are shown in Table 2 as follows:

Table 3.1 Equipments for arachidonic acid production and the usage

EQUIPMENT	USAGE
Gas chromatography	Fatty acid analysis
Incubator shaker	Cultivation and fermentation
Incubator	Cultivation and fermentation
Centrifuge	Solid-Liquid separation
Autoclave	Sterilization
pH meter	pH adjustment
Laminar flow hood	Culture transfer

3.1.0 Autoclave

In culturing the fungi, it is important to prevent any contamination. Autoclave was used to sterilized media and equipment required for growing microorganisms. Basic chemistry principle is used in autoclave, that is when pressure increase, the temperature also increase proportionally. Air was expelled from the autoclave and the pressure rose to 15 psi. With attained temperature 121 °C, all organisms were killed in 15 minutes. Figure 3.1 illustrates the autoclave (PH PMD 287, Hirayama) from Japan.

Figure 3.0: Autoclave model PH PMD 287

3.1.1 Laminar Flow Hood

Laminar flow hoods are provided with an atmosphere of filter to prevent the pure culture of microorganism from contamination during inoculation or transfer, at the same time, protect the researcher against infectious agents. In the beginning of the experiment, the ultraviolet light button of the flow hood is on for about 20 to 30 minutes to sterilize the inner part of it. After the cover of the flow hoods is taken away, the air flow button has to be on immediately. Then, researchers have to swab the surface of the inner hood with alcohol to prevent any contamination to the specimen. Operations, which are done in the hoods, are carried out in the vicinity of a Bunsen burner flame to decontaminate surfaces that are briefly exposed. Figure 3.2 shows the laminar flow hood model AHC-4A1.

Figure 3.1: Laminar flow hood model AHC-4A1

3.1.2 Incubator

Incubator was used to provide the desired temperature, ventilation and humidity for the growth of culture. The temperature and humidity levels were set for healthy growth of the cells after placing the culture in the incubator. Within a few days, the cells multiplied in number. The incubator is heated electrically or through water jet circulating over the outer phase of the incubator box. Hence suitable heat was passed to the samples in the laboratory incubator. Microbiological incubator (Memmert Model BE600 is illustrated in Figure 3.3.

Figure 3.2: Microbiological incubator model BE600

3.1.3 Incubator Shaker

Incubator shaker is equipped with shaking function which is needed for growing culture in liquid form. It offers quiet, maintenance-free shaking under a constant and reproducible temperature environment. Agitation speeds affect aeration and mixing of the culture. The greater the aeration the greater the oxygen transfer rate will be, and hence, increasing the cell growth rate. Figure 3.4 shows incubator shaker (Ecotron 100000, Infors) from Switzerland.

Figure 3.3 Incubator shaker model Ecotron 100000

3.1.4 Centrifuge

A centrifuge is a device that separates particles from suspensions according to their size, shape and density. In a solution, particles whose density is higher than that of the solvent sink, and particles that are lighter than it float to the top. The material to be centrifuged was distributed into centrifuge tubes which attached in a symmetric manner to a rotating block called the rotor. When the centrifuge tubes are spun, the centrifugal action creates an induced gravitational field in an outward direction relative to the axis of rotation and this drives the precipitated matter towards the bottom of the tube. Figure 3.5 illustrated the centrifuge (5810 R, Eppendorf) which is from Germany.

Figure 3.4 Centrifuge 5810 R

3.1.5 pH Meter

The pH meter is an electronic device used to measure the pH of the production medium before autoclaving. A typical pH meter consists of a special measuring probe where a glass electrode is connected to an electronic meter that measures and displays the pH reading.

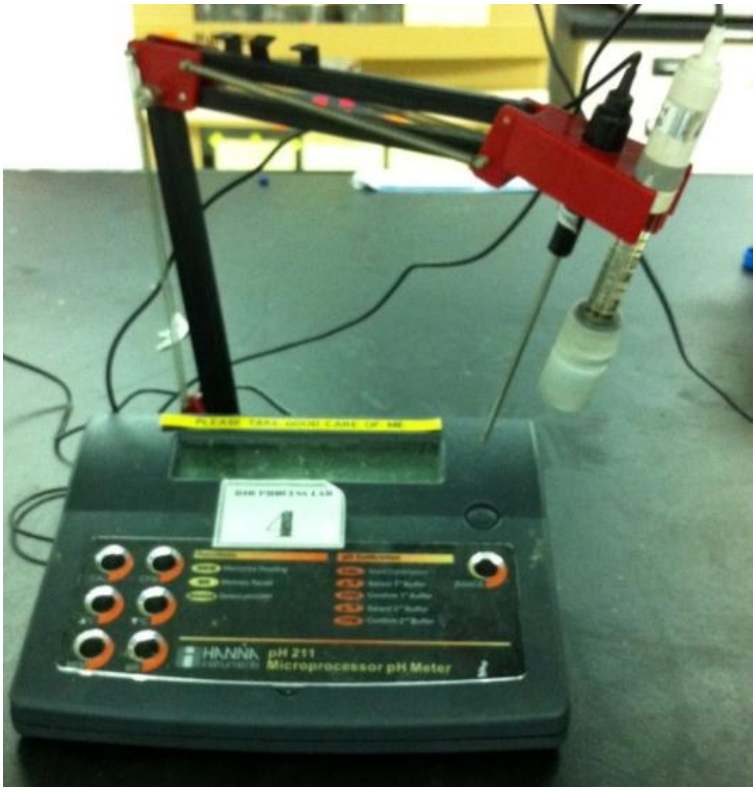

Figure 3.5 pH meter

3.1.6 Gas Chromatography

The gas chromatograph separates the volatile components of a very small sample and determines the amount of each component present. Mobile phase and stationary phase were required in running chromatography. The mobile phase or carrier gas comprised of nitrogen. The stationary phase consists of capillary column (50 m by 0.32 mm). The components in the sample can be separated because of interaction between the compound and the stationary phase. The stronger the interaction, the shorter time it will take to be spent in mobile phase, and the longer time it will move through the column. Hence, the retention time will be longer. The component in the sample will exit the column and flow past the detector. The detector shows different response with different component. Flame ionization detector is the most widely used detectors for organic sample. Figure 3.7 shows gas chromatography Agilent Technologies 6890 Series from USA.

Figure 3.6 Gas chromatography Agilent Technologies 6890 Series

3.2 Procedures

3.2.0 Experimental Design

The experimental design of this study was designed using Design of Experiment software where the varied parameters were glucose, potassium nitrate, potassium phosphate monobasic and glutamate. The experimental design is shown in Table 3.2.

Table 3.2 Experimental design

No	Glucose (g/L)	KNO_3 (g/L)	KH_2PO_4 (g/L)	Glutamate (g/L)
1	80	5	5	0
2	80	5	1	0
3	80	1	5	0.8
4	80	1	1	0.8
5	80	3	3	0.4
6	10	5	1	0.8
7	10	1	5	0
8	10	5	5	0.8
9	10	1	1	0
10	10	3	3	0.4
11	45	1	3	0.4
12	45	5	3	0.4
13	45	3	1	0.4
14	45	3	5	0.4
15	45	3	3	0
16	45	3	3	0.8
17	45	3	3	0.4
18	45	3	3	0.4
19	45	3	3	0.4
20	45	3	3	0.4
21	45	3	3	0.4

3.2.1 Overall Process Flow

The overall process flow of arachidonic acid production is shown in Figure 3.8 below.

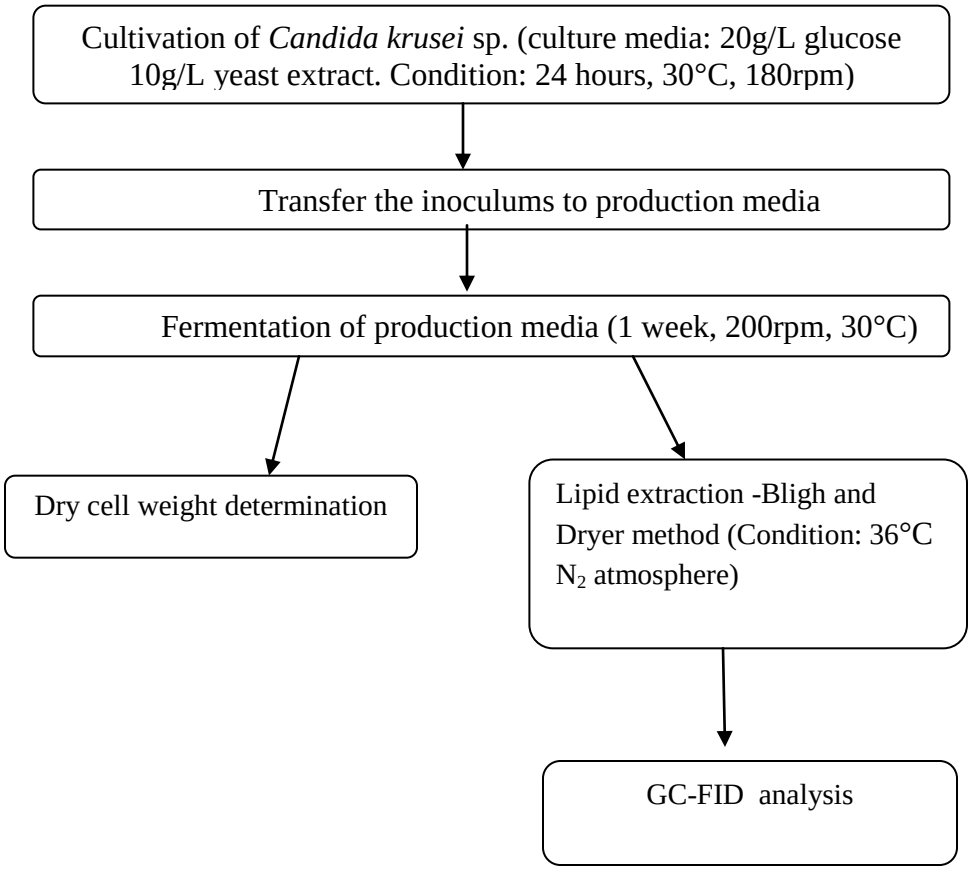

Figure 3.7 Process flow chart of ARA production

3.2.2 Growth Medium

Growth media was prepared by 2 g of glucose and 1 g of yeast extract added to 100 ml of distilled water and was autoclaved for 20 minutes at 121°C. The colony of *Candida krusei* was transferred using inoculating loop from the culture plate into the flasks containing growth media. Sterilization was taken during the transferring of *Candida krusei*. The medium was then placed in the incubator shaker for 24 hr at 36°C with 180 rpm agitation.

3.2.3 Production media

The condition of production media was varied so that the optimum condition can be studied. The composition of production media consists of (g/L): carbon source, 30; yeast extract, 5.0; KNO_3, 1.0; KH_2PO_4, 2.4; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; $FeCl_3.6H_2O$, 0.015; $ZnSO_4.7H_2O$, 0.0075; $CuSO_4.5H_2O$, 0.005 and mixed with distilled water. For the effects of glutamate on the ARA production, 0, 0.2, 0.4, 0.6, 0.8 g/l glutamate will be respectively added to the production medium. (Yu et al., 2002). All the fermentation experiments were performed in 500 ml Erlenmeyer flasks containing 200 ml of basal fermentation medium according to the experimental design. The carbon sources used is glucose. The pH of media was adjusted by using 0.5 M hydrochloric acid (HCl) and 0.5 M sodium hydroxide (NaOH) to 6.5 before autoclaving. After autoclaved, the media was inserted with 10% of grow media and incubated for 7 days at 30°C. The agitation speed was set to 200rpm.

3.2.4 Dry cell weight determination

The empty centrifuge tubes were weighed. Each sample was centrifuged and the supernatant were discarded. Biomass production is determined by harvesting the cells by suction filtration followed by drying at 55–60°C until a constant weight is attained (Nisha et al,2010). The remained biomass was weighed together with the centrifuge tube. The total weigh of biomass was calculated.

3.2.5 Lipid extraction

Lipid was extracted by the two-step Bligh and Dyer (1959) method. Firstly, the cell was disrupted by using ultrasonic bath. 1 ml of cell suspension was taken and was put into a centrifuge tube containing 3.75 ml of a mixture of chloroform: methanol with the ratio 1:2 respectively. The mixture was then vortex for 5-10 min. After that, 1.25 ml of chloroform was added with 1 minute of mixing, and 1 ml of distilled water was added followed by 1 minute of mixing. Then, the sample was centrifuged for 15 min at 8000 rpm. Subsequently, the lower phase was collected in another tube. About 1.88 ml of chloroform was added to the non-lipid residue and was vortex before centrifuged at 8000rpm for 15 minutes. The lower phase was mixed with the upper phase from the first centrifugation. The lipid extract was evaporated until it was about to dry, and dissolved in a small volume with a ratio of chloroform: methanol (2:1). Lastly, the sample will be filter using 0.22 µm Nylon filter and place in vials for further analysis. For a clear view of process of lipid extraction, the process flow is summarized as in Figure 3.10

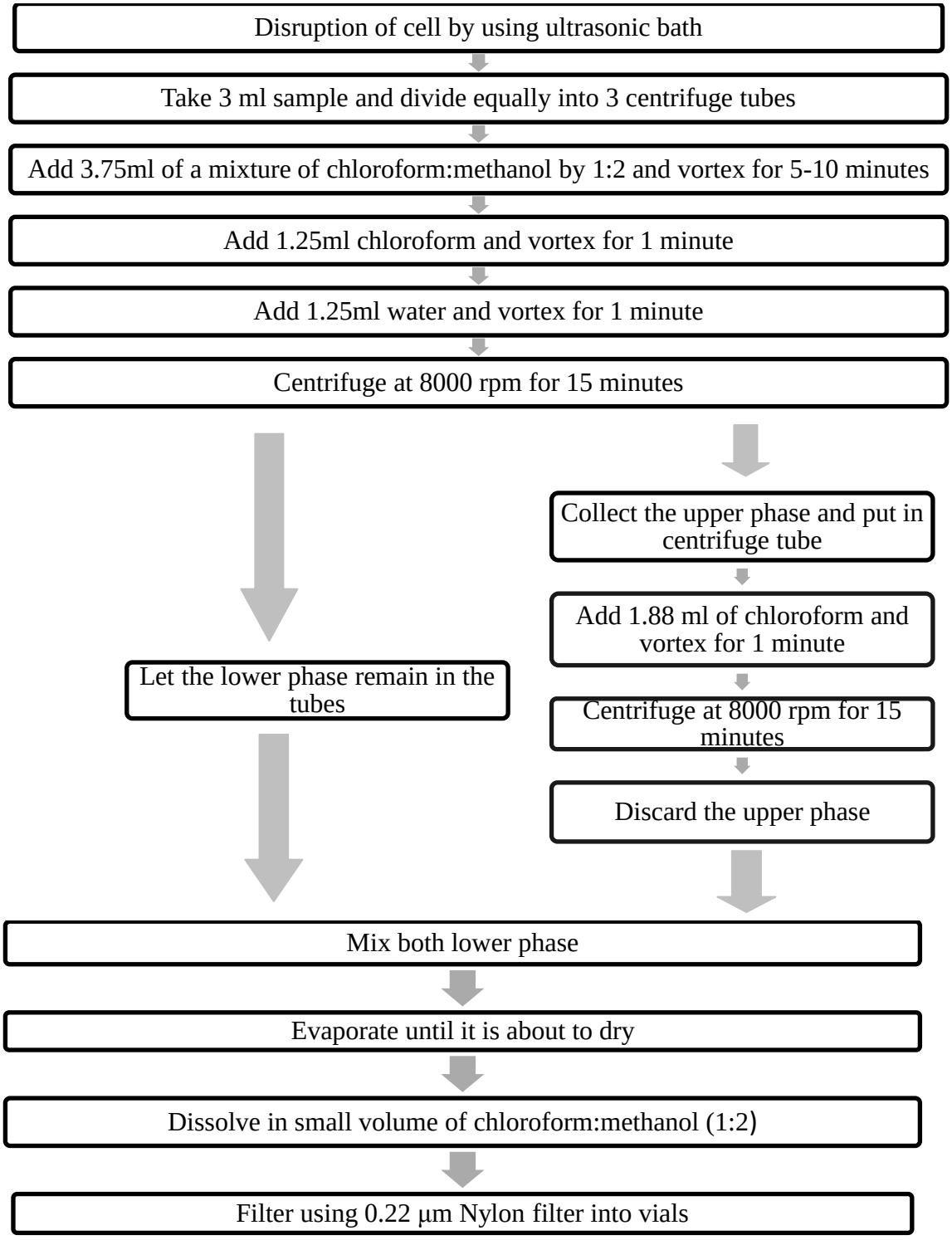

Figure 3.8 Process flow of lipid extraction

3.2.6 Biomass and fatty acids analysis

The total fatty acid composition of the lipids was determined by gas chromatography (GC) with capillary column 0.25 mm x 30 m x 0.25 µm, a flame ionization detector, using helium as the carrier gas. An aliquot (2 µl) of each sample extract was injected onto the GC column using the injector in the split mode. The initial column temperature was 185 °C (0.5 min) and this was increased at a rate of 3.5 °C min^{-1} to 235 °C (14.3 min), and then will be maintained at 235 °C for 1.0 min. ARA methyl ester was then will be identified by comparing the retention time of the methyl ester of an ARA standard. The amount of fatty acid was estimated from peak areas compared with calibration standards. (Saelao et al., 2010).

CHAPTER 4

RESULT AND DISCUSSION

4.0 Subculture of *Candida krusei*

Subculture is an essential procedure in order to revive the cells from its inactive state to obtain pure colony. The agar cultures were placed in an incubator at temperature 36 °C. After a few days, there was no growth of cells in some agar plates. This problem occurred probably because the inoculating loop was still hot after flamed when picking and scraping off the colony of the bacteria where the hot loop might have killed the bacteria. In order to solve this problem to happen, the inoculating loop should be cooled first by stabbing it into the agar in a spot that does not contain a bacterial colony. However, there was still bacterial growth obtained in some agar culture. The growth of the bacteria can be seen in Figure 4.0.

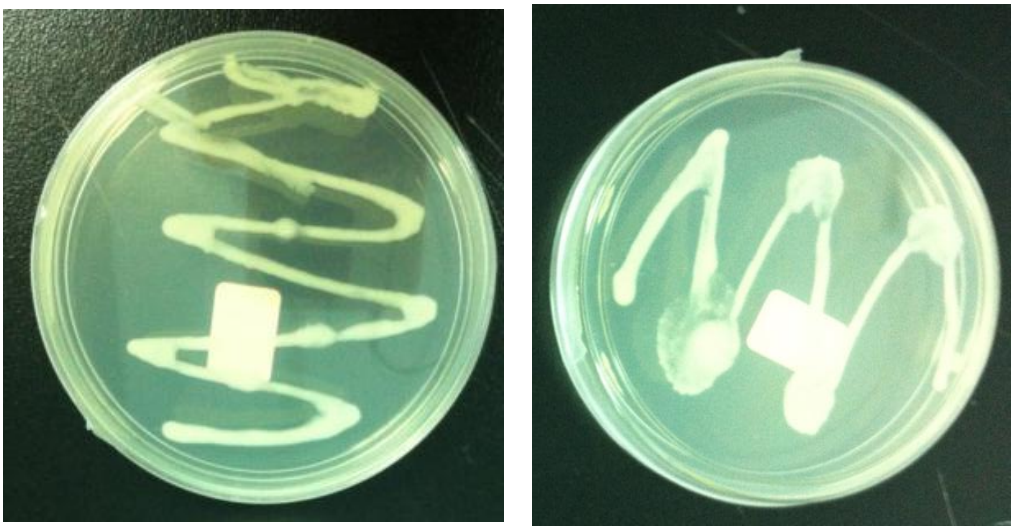

Figure 4.0 *Candida krusei* sp. culture on agar plates after 4 days at 36 °C.

4.1 Culture of *Candida krusei* in growth medium

The *Candida krusei* was inoculated in growth medium before inoculated in production medium. The growth medium which called inoculums supports the growth in liquid form. When inoculating bacterial in growth medium, it is essential to cover the flask used with cotton dressing. Cotton dressing was used to filter for sterile gas exchange and as a barrier against contaminations. The medium was left in incubator shaker with agitation speed 180 rpm and 30 °C for 24 hours. However, for the first trial of inoculating, the cell wall growth did not appear and the medium was slightly changed in color. This may due to some error during transferring the bacteria from agar plate to the growth medium where the inoculating loop used was not cooled before picking the bacteria. In order to avoid same mistake to occur, inoculating loop have to be cooled

first. The culture of *Candida krusei* and change in color of the medium is shown in Figure 4.1.

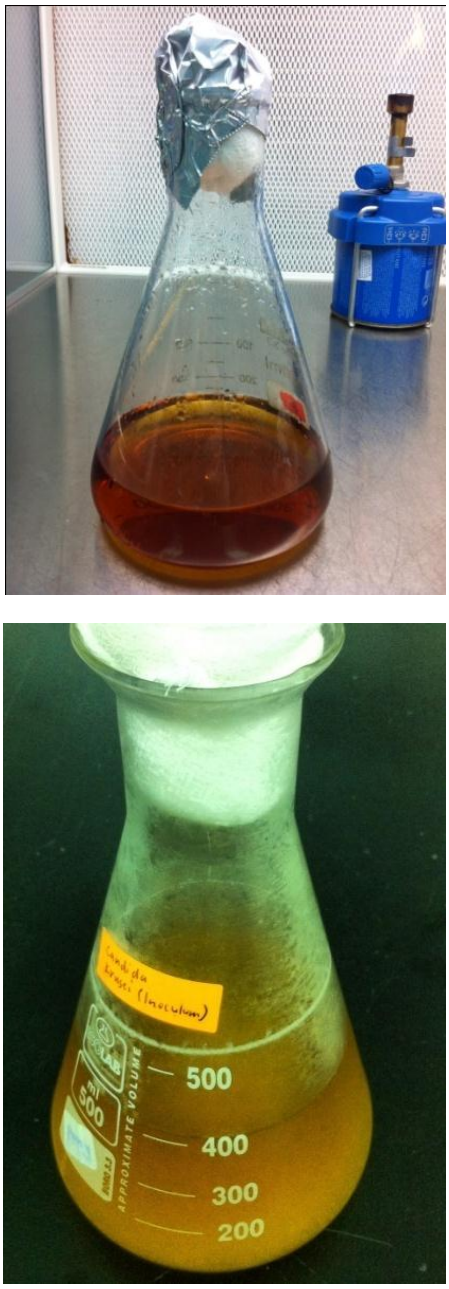

Figure 4.1 A.Growth medium (20g/L of glucose and 10g/L of yeast extract) after autoclave. B. Growth medium after cultivation for 24 hours at 36 °C with 180 rpm agitation speed.

4.2 Culture of *Candida krusei* in production medium

Production medium provides supplements for the production of desired product. Different view was obtained in different medium condition. Based on the observation, cell wall growth was obtained in all the samples. However, the cell wall growth appeared thickest when high amount of glucose, 80 g/L was supplied and inversely the cell wall growth appeared unclear when 45 g/L of glucose supplied. These showed that the growth of the bacteria is high when higher amount of glucose is supplied. A clearer view of the appearance of cell wall growth is shown in Figure 4.3.

A B

Figure 4.2 *Candida krusei* culture in different amount of glucose in production medium after one week of cultivation at 30 C and agitation speed of 200rpm. A Thick cell wall growth in 80 g/L glucose; B Unclear cell wall growth in 45 g/L glucose

4.3 Data analysis result

Standard Arachidonic acid (ARA) was diluted into five different concentration which are 0.01 g/ml, 0.02 g/ml, 0.03 g/ml, 0.04 g/ml and 0.05 g/ml. A peak was obtained in all the analysis of the concentration except for 0.01 g/ml of standard ARA where no peak appeared. Based on the chromatogram result, the retention time were approximately around 14.737 minutes. The calibration curve was plotted and the ARA yield was calculated. The result for every design was summarized in Table 4.2 below.

Table 4.1 Total Biomass and arachidonic acid (ARA) yield

No	Glucose (g/L)	KNO_3 (g/L)	KH_2PO_4 (g/L)	Glutamate (g/L)	Total biomass (g/mL)	AA Yield (g/ml)
1	80	5	5	0	0.0661	0.5040
2	80	5	1	0	0.0641	0.2085
3	80	1	5	0.8	0.0701	0.1734
4	80	1	1	0.8	0.0720	0.1686
5	80	3	3	0.4	0.0677	0.1665
6	10	5	1	0.8	0.0691	0.1654
7	10	1	5	0	0.0721	0
8	10	5	5	0.8	0.0590	0
9	10	1	1	0	0.0643	0
10	10	3	3	0.4	0.0626	0
11	45	1	3	0.4	0.0777	0
12	45	5	3	0.4	0.0751	0
13	45	3	1	0.4	0.0719	0
14	45	3	5	0.4	0.0719	0
15	45	3	3	0	0.0772	0
16	45	3	3	0.8	0.0790	0
17	45	3	3	0.4	0.0705	0
18	45	3	3	0.4	0.0750	0
19	45	3	3	0.4	0.0752	0
20	45	3	3	0.4	0.0800	0
21	45	3	3	0.4	0.0753	0

4.4 Effect of potassium nitrate (KNO_3)

Based on the ARA yield obtained, ARA yield was at the highest, 0.504 g/l, when high concentration of potassium nitrate was supplied. As suggested by Chen et al. (1997) who worked on Wuji-H4 isolate (M. alpina strain), he concluded that potassium nitrate to be superior to the ammonium salts. The effect of KNO3 on ARA yield and total biomass can be obtained in Figure 4.5.

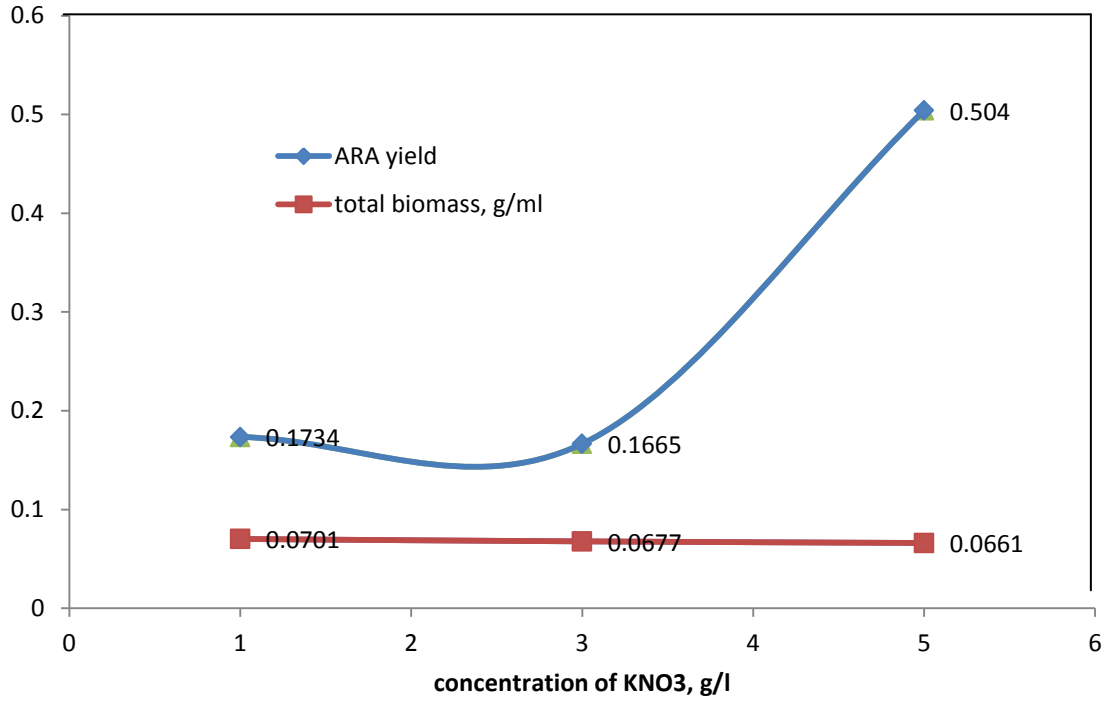

Figure 4.3 Effect of KNO_3 on ARA yield and total biomass in 1 g/l, 3 g/l and 5 g/l of KNO_3 after 7 days at 30°C and agitation speed 200 rpm

However, the total biomass was decreasing with increasing concentration of KNO_3. Similarly, decreasing in nitrate supplied caused ARA yield to decrease. As suggested by Yu et al, ARA yield decrease because the depletion of nitrogen in the growth medium evokes a decrease in the intracellular concentration of an activator of isocitrate dehydrogenase. Hence, as a consequence, an increase in the contents of citrate and isocitrate in the cells occur. In this matter, it is a function of the amount of carbon source provided and that lipid accumulation commences at the onset of nitrogen depletion. When feed concentration of glucose was below 80 g/l, glucose needed for bacterial growth was inefficient. Therefore, no matter how much nitrate was fed, bacterial growth to produce ARA was still relatively poor.

4.5 Effect of potassium phosphate monobasic (KH_2PO_4)

The highest ARA yield produced was when higher concentration of KH_2PO_4 was supplied, 0.504 g/ml and the yield decreasing when lesser KH_2PO_4 was supplied. The trend of the total of biomass obtained was almost similar with what obtained in KNO3 where there was no drastic drop or plunge on the total biomass. Based on Figure 4.6, the relationship of total biomass and ARA yield can be observed. The ARA yield obtained increase with increasing total biomass. This explained that the addition of KH_2PO_4 caused filamentous growth and increased viscosity where the addition of phosphorus affected the lipid composition. While, at high carbon/nitrogen ratios, the biomass production is decreasing with increasing KH_2PO_4. KH_2PO_4 provided the nitrogen flux

which is crucial for a high biomass buildup but could not accelerate the production of total lipids which occur in nitrogen depleted media. A decrease in total fatty acid content is attributed to a differential increment in cell division and subsequent reduction in the rate of lipid synthesis.

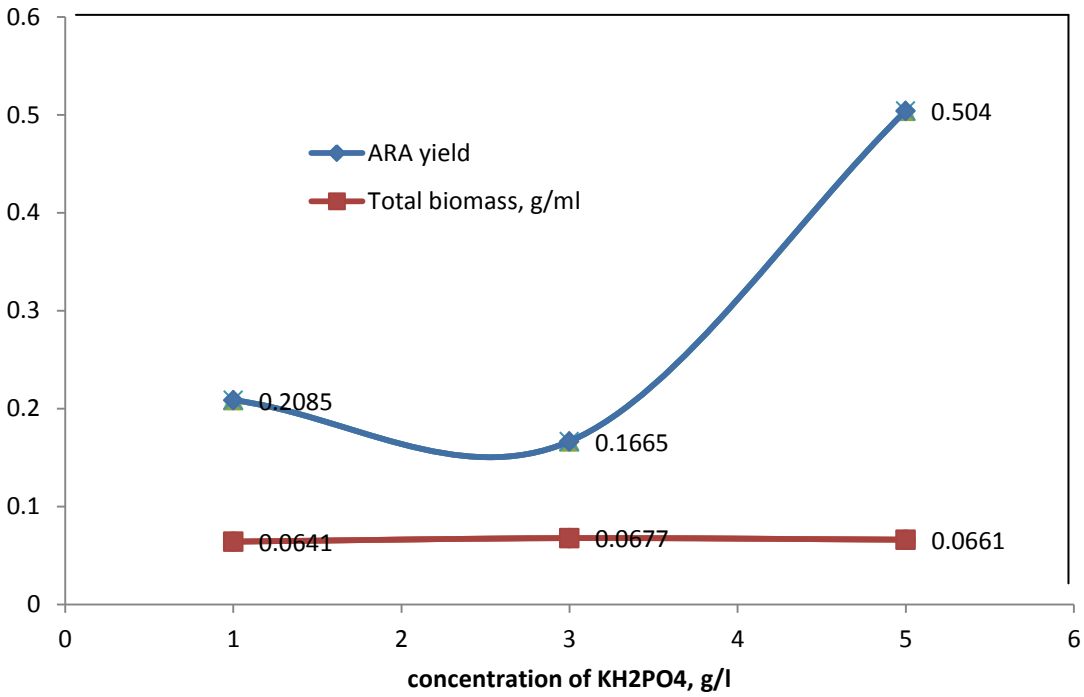

Figure 4.4 Effect of KH_2PO_4 on ARA yield and total biomass in 1 g/L, 3 g/L and 5 g/L of KH_2PO_4 after 7 days at 30°C and agitation speed 200 rpm

4.6 Effect of glutamate

Based on the result, ARA yield of was significantly lower when high concentration of glutamate was supplied where ARA yield is 0.1686 g/ml. This was an evidence that 0.8 g/L glutamate could not improve arachidonic acids production from the *Candida krusei*. However, this result was an inverse with what suggested by Yu et al. where he mentioned that the application of the 0.8 g/L glutamate could be useful in developing rational strategies for enhancing the production of ARA in large-scale fungal *M. alpina* cultures. Glutamate is involved in nitrogen metabolism and is required as an essential precursor of protein and nucleotide synthesis as well as a substrate for energy metabolism in the organism. However, glutamate did not affect the substantial influence on cell growth in this study. The effect glutamate on ARA yield and total biomass can be obtained in Figure 4.7.

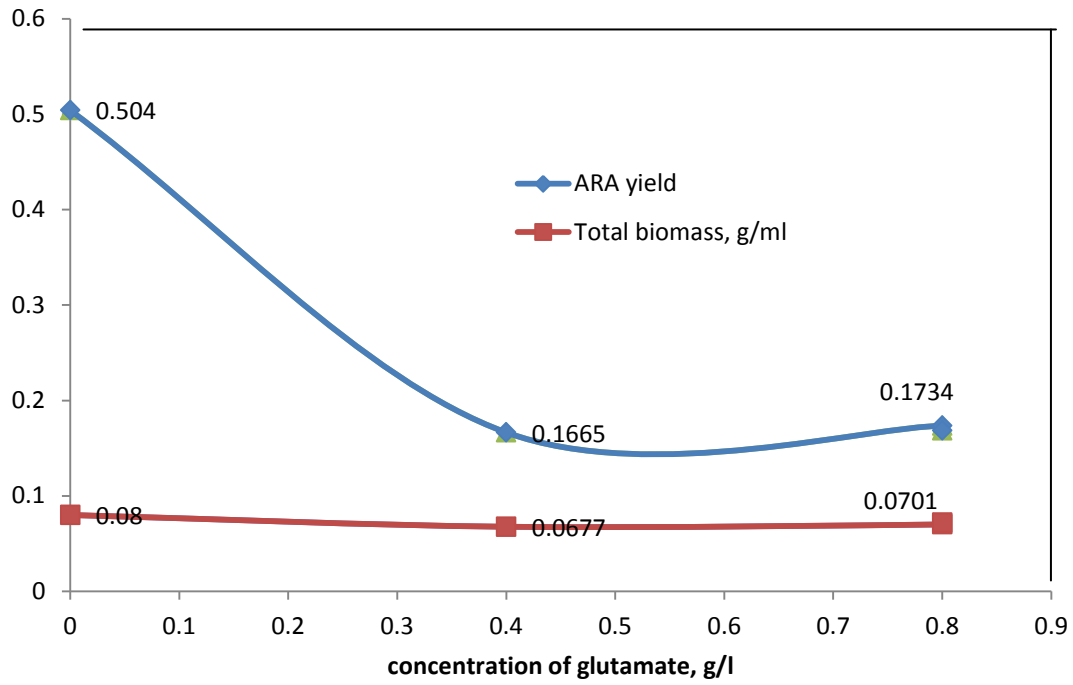

Figure 4.5 Effect of glutamate on ARA yield and total biomass in 0 g/L, 0.4 g/L and 0.8 g/L of glutamate after 7 days at 30°C and agitation speed 200 rpm

4.7 Effect of glucose

The highest production yield of arachidonic acid (ARA) was 0.504 g/L which was when 80g/L of glucose was supplied. This may be because Candida krusei species prefer to grow rapidly and proliferate extensively on simple sugars. According to dynamic analysis, higher initial glucose concentration impaired bacterial growth at early days of cultivation and low initial concentration of glucose was good to shorten the lag phase of bacterial growth after inoculation. However, based on the result obtained from

this study, when glucose concentration was decreased, which are at 10 g/L and 45 g/L of glucose, the ARA yield was also decreasing. This probably because of when residual glucose is low, bacterial stopped growing because the nutrition that keeps the bacteria growing was decreasing and insufficient. Furthermore, we can see that ARA yield was increasing with increasing total biomass. For the commercial production of ARA, a high biomass concentration is required for high productivity because ARA is an intracellular product.

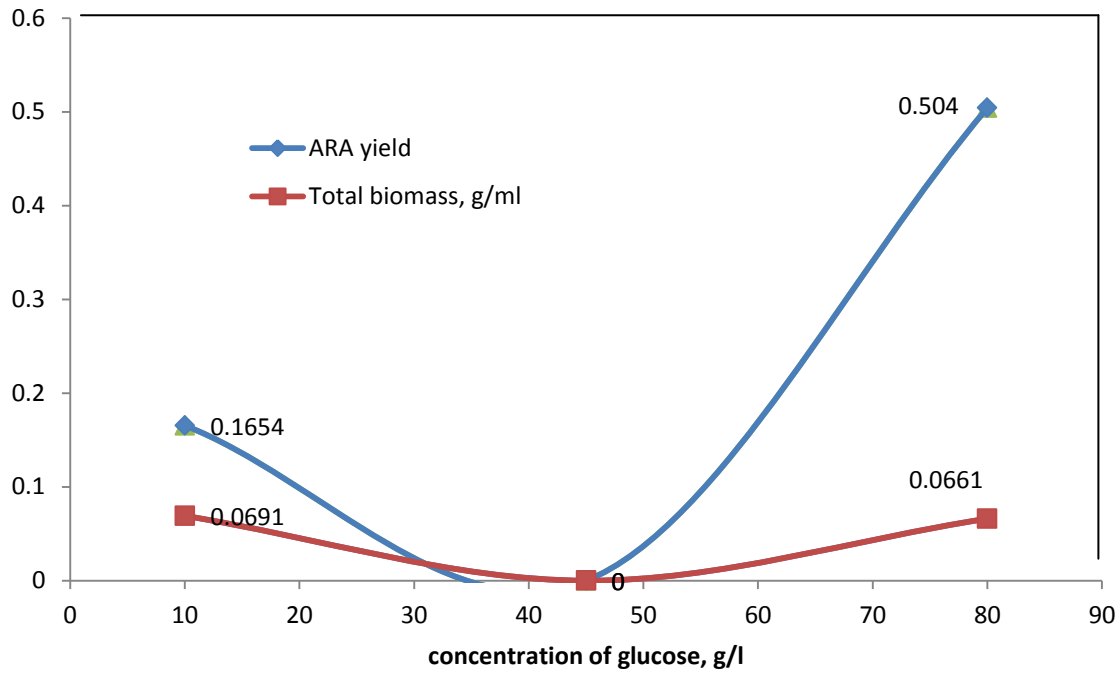

Figure 4.6 Effect of glucose on ARA yield and total biomass in 10 g/L, 45 g/L and 80 g/L of glucose for 7 days at 30°C and agitation speed 200 rpm

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.0 CONCLUSION

In conclusion, to achieve the objectives of the study, the highest total of biomass produced was when glucose concentration is 45 g/L, KH_2PO_4, 3.0 g/L, KNO_3, 3.0 g/L and glutamate, 0.4 g/L but the highest yield of ARA, 0.504 g/L obtained was when glucose concentration is 80 g/L, KH_2PO_4, 5.0 g/L, KNO_3, 5.0 g/L and no glutamate. Besides, when no glutamate supplied, the yield was increasing with increasing concentration of glucose.

Besides that, it was also proved that respond surface method (RSM) is one of the best methods to optimize variables to achieve the highest production of arachidonic acid (ARA). Furthermore, this study also proved that alternatives source of ARA production which are by fermentation of *Candida krusei* is one way to produce a Halal standard ARA which is suitable to be consumed by all types of community.

Apart from that, the production from *Candida krusei* shows a good advantage over other sources as the production process can be completed in shorter time. Production of ARA from *Candida krusei* is also easier compared to other sources as it can be obtained easily. Besides that, the process is less complicated compared to animal source, which contain cholesterol and objectionable tastes and odors that need to be removed first.

In a long run, the wide application of arachidonic acid (ARA) can guarantee the potential market place as well of human's health.

5.1 RECOMMENDATION

Limitations of equipment in the laboratory makes the whole project takes longer time than expected. Equipments such as incubator, autoclave and gas chromatography were continuously used by other users. The unavailability of certain chemicals also had delayed the project. Better result might be obtained if the problems faced can be solved earlier.

Further studies on the media components in arachidonic acid (ARA) production should be directed at varying other significant parameters including nutrients needed and method of extraction. Other method of extraction such as Soxlet method might be more complicated but the production yield might be higher. There are other parameters that must be considered and should not be ignored. Production of a fatty acid is a difficult process and hence suitable method must be used. Long time duration is needed in order to succeed in producing ARA.

Candida krusei has been used in this study to produce ARA. There are many more microbial organisms such as *Aureispira maritima* and *Mortierella alpina* where these species of microbes are also producing ARA. Different microbes may produce different yield of ARA and by using different microbes, we can determine which microorganism can produce high yield of ARA in same varied parameters used in this study.

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APPENDIXES

APPENDIX A.1

The data of the sample concentration for each sample is shown in Table A.1.

Table A.1 Raw data for sample concentration

Run	Mass of empty tube (g)	Mass of freeze-dried sample tube (g)	Dry biomass tube (g)	Sample concentration (g/mL)
1	9.7461	12.7189	2.9728	0.0661
2	9.8767	12.7604	2.8837	0.0641
3	9.8665	13.0205	3.154	0.0701
4	9.7892	13.0278	3.2386	0.0720
5	9.9874	13.0338	3.0464	0.0677
6	9.8827	12.9932	3.1105	0.0691
7	9.7583	13.0043	3.2460	0.0721
8	9.9583	12.6130	2.6547	0.0590
9	9.8501	12.7439	2.8938	0.0643
10	9.8847	12.7037	2.8190	0.0626
11	9.9675	13.4651	3.4976	0.0777
12	9.9274	13.3068	3.3794	0.0751
13	9.8578	13.0975	3.2397	0.0719
14	9.7696	13.0045	3.2349	0.0719
15	9.8973	13.3714	3.4741	0.0772
16	9.8633	13.4176	3.5543	0.0790
17	9.7775	12.9499	3.1724	0.0705
18	9.8611	13.2364	3.3753	0.0750
19	9.8603	13.2433	3.3830	0.0752
20	9.7099	13.3051	3.5952	0.0800
21	9.7121	13.1041	3.3920	0.0753

APPENDIX A.2

Calculation for dry biomass.

Condition 1:

Glucose - 80 g/l

KNO_3 - 5 g/l

KH_2PO_4 – 5 g/l

Glutamate – 0 g/l

Mass of empty tube = 9.7461g

Mass of sample from tube = (12.7189 - 9.7461) g

= 2.9728 g

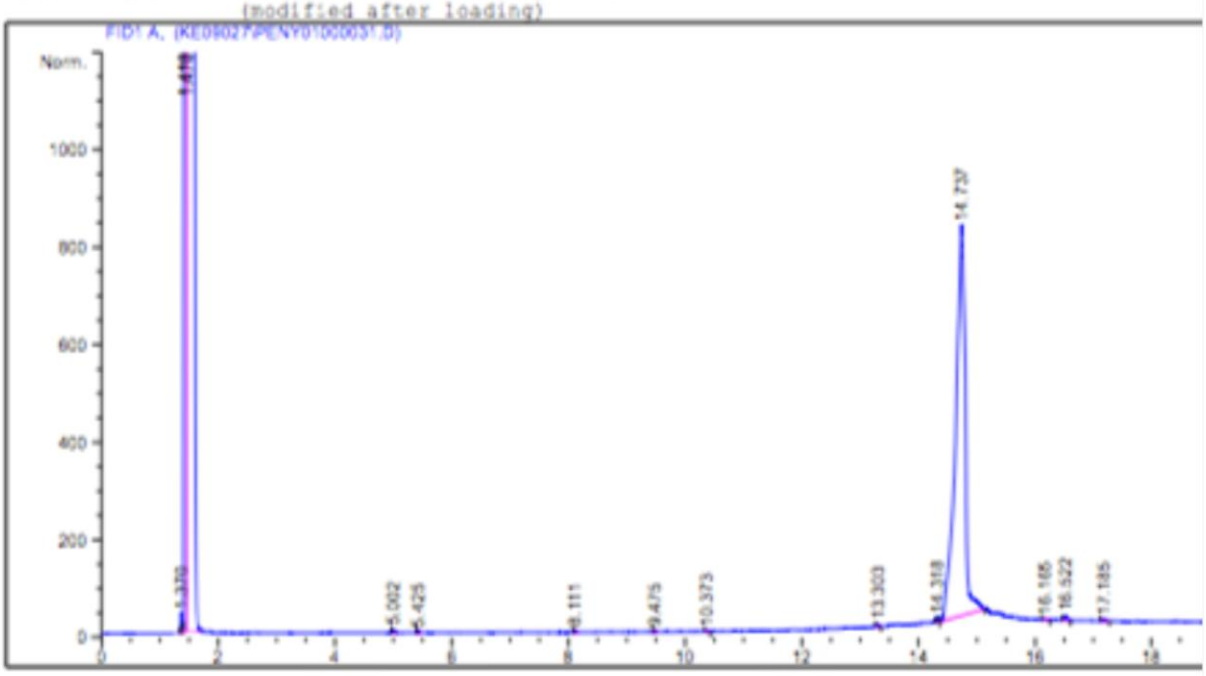
So, total biomass = 2.9728 g

APPENDIX B

Gas chromatography data

The gas chromatography data is shown in Figure B.1 where the peak represented the presence of standard arachidonic acid.

```
Acq. Operator   : AMIRUL                          Seq. Line :   5
Acq. Instrument : Instrument 1                     Location : Vial 5
Injection Date  : 1/17/2013 1:16:57 AM                 Inj :   1
                                                  Inj Volume : 1 µl
Acq. Method     : C:\CHEM32\1\METHODS\FREE FATTY ACID INNOWAX.M
Last changed    : 1/16/2013 11:17:33 AM by AMIRUL
Analysis Method : C:\CHEM32\1\METHODS\ALCOHOL BUTANOL.M
Last changed    : 1/17/2013 11:03:07 AM by AMIRUL
                  (modified after loading)
```




```
================================================================================
                            Area Percent Report
================================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: FID1 A,

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [pA*s]      [pA]       %
----|-------|----|-------|----------|----------|--------|
   1   1.370 BV    0.0264   66.59347   40.12531  0.02365
   2   1.419 VV S  0.0382 1.06332e5  4.64019e4  37.77067
   3   1.476 VB S  0.0603 1.65916e5  4.58427e4  58.93556
   4   5.002 BB    0.0294   11.44683    5.97455  0.00407
   5   5.425 BB    0.0300    4.64337    2.35866  0.00165
   6   8.111 BB    0.0282    7.16335    3.94598  0.00254
   7   9.475 BB    0.0275    3.73190    2.12365  0.00133
   8  10.373 BB    0.0364    5.62043    2.39060  0.00200
   9  13.303 BB    0.0340   12.50827    5.41052  0.00444
  10  14.318 BV    0.0289   18.33225    9.75635  0.00651
  11  14.737 VB    0.1433 9091.67480  801.48859  3.22949
  12  16.165 BB    0.0496    8.81865    2.66283  0.00313
  13  16.522 BB    0.0491   31.01880    9.75797  0.01102
  14  17.185 BB    0.0521   11.09207    3.22844  0.00394
```

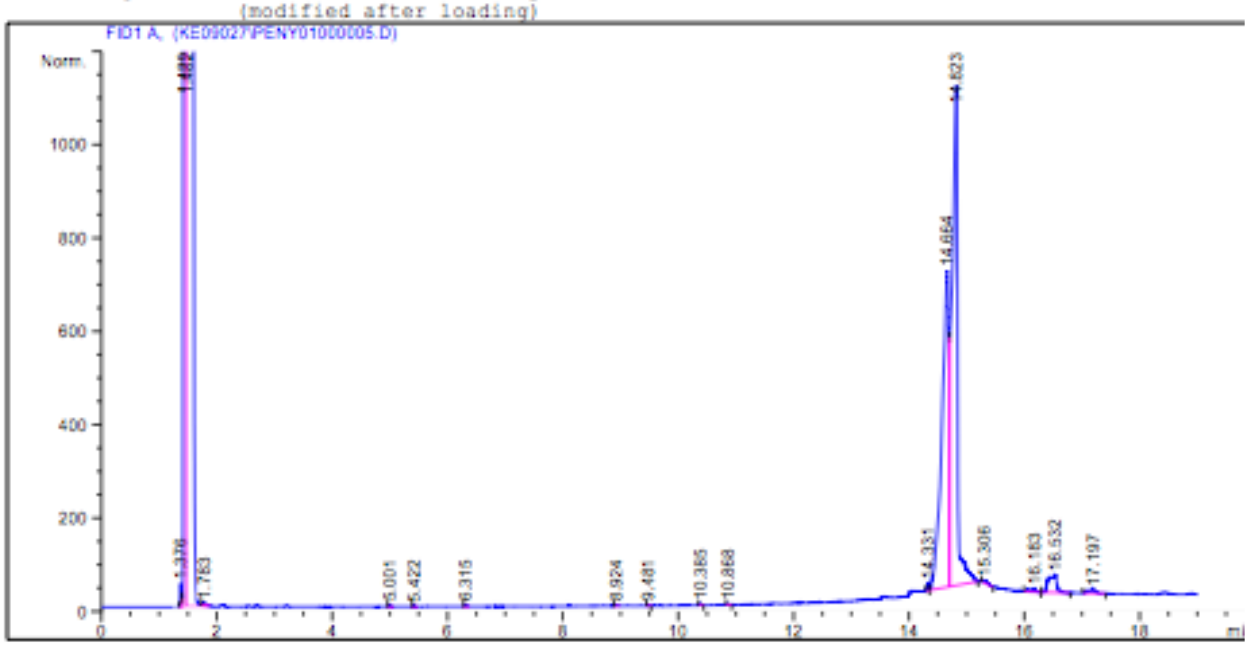
Figure B.1 Data analysis for standard Arachidonic Acid by gas chromatography

APPENDIX C

The gas chromatography data is shown in Figure C.1 where the peak represented the presence of standard arachidonic acid in the sample.

Data File C:\CHEM32\1\DATA\KE09027\PENY01000005.D
Sample Name: 1

Acq. Operator	: AMIRUL	Seq. Line	: 1
Acq. Instrument	: Instrument 1	Location	: Vial 1
Injection Date	: 1/16/2013 3:01:36 PM	Inj	: 1
		Inj Volume	: 1 µl
Acq. Method	: C:\CHEM32\1\METHODS\FREE FATTY ACID INNOWAX.M		
Last changed	: 1/16/2013 11:17:33 AM by AMIRUL		
Analysis Method	: C:\CHEM32\1\METHODS\ALCOHOL BUTANOL.M		
Last changed	: 1/17/2013 11:03:07 AM by AMIRUL (modified after loading)		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	1.376	BV	0.0275	87.60108	49.83335	0.04048
2	1.429	VV S	0.0266	4.76790e4	2.69651e4	22.03487
3	1.482	VB S	0.0570	1.54966e5	4.53068e4	71.61761
4	1.783	BB X	0.0572	18.17058	5.27668	0.00840
5	5.001	BB	0.0297	4.68534	2.40930	0.00217
6	5.422	BB	0.0293	2.87596	1.50204	0.00133
7	6.315	BB	0.0280	5.79245	3.21090	0.00268
8	8.924	BB	0.0386	5.04600	1.99156	0.00233
9	9.481	BB	0.0265	3.90779	2.33493	0.00181
10	10.385	BB	0.0315	7.43557	3.54307	0.00344
11	10.868	BB	0.0344	5.11089	2.25622	0.00236
12	14.331	BV	0.0280	28.63047	15.89905	0.01323
13	14.664	VV	0.1086	5614.26025	678.06958	2.59463
14	14.823	VB	0.0958	7424.61328	1071.52222	3.43129

Figure C.1 Data analysis for standard Arachidonic Acid in sample by gas chromatography

APPENDIX D

Table of analysis data of samples by gas chromatography is shown in Table D.1

Table D.1 Analysis data of samples

RUN	AREA	AA YIELD (g/ml)
1	12896.2	0.504
2	1685.2	0.2085
3	356.9	0.1734
4	173.1	0.1686
5	93	0.1665
6	50.8	0.1654
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0
16	0	0
17	0	0
18	0	0
19	0	0
20	0	0
21	0	0